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Evaluation of antimicrobial activity of ellagic acid on Methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, and *Escherichia coli*

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Abstract---Serious infections, and rising resistant infections, by pathogenic bacteria, have become developing threats worldwide. There is an increasing need to develop and discover a new treatment options that can act effectively to cure infections, with lesser resistance, side effects and toxicity. Identification of new treatment options against pathogens is an important issue nowadays. Ellagic acid is a non-drug compound that has been shown to possess many biological activities and is presented in many red fruits and berries. This study aims to evaluate and measure the antimicrobial activity and the minimum bactericidal concentration (MBC) of ellagic acid on Methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, and *Escherichia coli*. The antibacterial activity of ellagic acid was evaluated against ten purified bacterial isolates via agar disc diffusion assay, and minimum bactericidal concentration assay (MBC). The results show that ellagic acid was an effective antimicrobial compound against all the tested pathogens. The MBC range was shown to be between (1-2) mg/ml for MRSA, and *E.coli*, and between (1-1.5) mg/ml for *P. aeruginosa*. This current study has detected and proved the antimicrobial activity of ellagic acid against important and widely resistant pathogenic bacteria like MRSA, *P. aeruginosa*, and *E.coli*

Keywords---pathogenic bacteria, ellagic acid, antibacterial activity.

Introduction

Ellagic acid is a phenolic phytochemical present in many fruits and vegetables. It was first discovered by chemist Henri Braconnot in 1831 (Grasser and Enna, 1922). The highest levels of ellagic acid are found in raw chestnuts, walnuts, pecans, cranberries, raspberries, strawberries, and grapes, distilled beverages, peaches and pomegranates (Infante *et al.*, 2011; Usta *et al.*, 2013). It is found either in free form or as a part of complex compounds called ellagitannins, which can be metabolized to ellagic acid and many of its metabolites, including urolithins (Larrosa *et al.*, 2006). Urolithins are also gut flora human metabolites of dietary ellagic acid derivatives. Ellagic acid has low bioavailability, with 90% remaining unabsorbed from the intestines until metabolized by microflora to the more bioavailable urolithins (Luca *et al.*, 2020).

Ellagic acid has been shown to have many pharmacological activities. It has antioxidant activity either directly or by indirectly inducing cellular antioxidant enzyme systems (Indira *et al.*, 2002). Also has the ability to decrease the lipidemic profile and lipid metabolism (Devipriya *et al.*, 2008), inhibit NF- κ B activity and Erk1/2 activation and decreased NO and PGE2 synthesis, alter interleukin-1 β , interleukin-6 level, increase nuclear factor erythroid 2-related factor 2 expression (Ghadimi *et al.*, 2021; Ahad *et al.*, 2014; El-Shitany *et al.*, 2014). Ellagic acid has proved to have effects on insulin, glycogen, phosphatases, aldose reductase, sorbitol accumulation, advanced glycation end-product formation, and resistin secretion (Ghadimi *et al.*, 2021; Yoshimura *et al.*, 2013). All these molecular mechanisms leads to ellagic acid anti-atherogenic, anti-inflammatory, anticancer, neuroprotective, antidiabetic effects. Anticancer properties has been explained by several mechanisms including inhibition of tumor cell migration, apoptosis, modulating protein and gene expression, interfering with signaling pathways and angiogenesis. Also the sensitivity to chemotherapy and radiotherapy has been found to be increased with ellagic acid. (Ceci *et al.*, 2018; Vlachoianis *et al.*, 2015) To date, only few clinical researches supports and evaluate the EA chemopreventive and therapeutic potential. All these important pharmacological effects, makes ellagic acid an interesting natural phenolic compound that can be processed to be used in many health fields.

The study of antimicrobial capacity of plant phenolic compounds is important therapeutic field that held promises in having antimicrobial activity. Ellagic acid and ellagitannins have been studied for their effect against some types of bacteria and fungi. Antimicrobial resistance, is the main reason to go on discovering and developing a new antimicrobial compounds to overcome the resistance. Ellagic acid is a new natural phenolic compound that held the promise for its antimicrobial activity in addition to its other therapeutic benefits. This study aims to evaluate and measure the antimicrobial activity of ellagic acid on *Methicillin-resistant Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, and *Escherichia coli*. Also aims to determine the minimum bactericidal concentration for each of the tested bacteria.

Materials & Methods

Isolation, purification, Identification and activation of bacteria Strains

About 70-80 bacterial strains of the three bacterial types were obtained from clinical specimen: *Methicillin-resistant Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, and *Escherichia coli*. They were isolated using suitable medium; and identified by gram stain, colony morphology, biochemical tests (Coagulase test and catalase test) and API-20E test strip (from bioMerieux, Inc.). Ten selected purified strains of *S. aureus*, *P. aeruginosa*, and *E. coli* were chosen for the study, which mainly shown the highest values of resistance to traditional antimicrobial medication. Clinical specimens were acquired from the teaching laboratories of medical city hospital in Baghdad. A selective colony of each microorganism was transferred from their agar plates to 10ml of sterile Brain Heart Infusion Broth (BHI-B) and incubated for 24hrs aerobically at 37 °C. The purity of the isolates was checked. This process was done according to Beighton, 1985. To activate inoculums, 0.1 ml of the pure isolates was added to 10 ml of sterile BHI-B and followed by incubation aerobically for 18hrs. at 37°C for activation before each experiment (Fingold and Baron, 1986).

Resistant strains of bacteria to different traditional antimicrobial medications were determined by Disc diffusion test. Antibiotic susceptibility test was performed according to CLSI, (2015) to choose the most resistant strains for the study. All culture media were sterilized by autoclave at 121°C and pressure of 15 pound/inch² for 15 minutes. Sterilization of all cleaned glass wares was conducted by hot air oven at 180°C for 1 hr. Benches and floor of the laboratory were disinfected by bleaching antiseptic solution (Fas).

Preparation of Ellagic acid solution

Ellagic acid ≥96% powder from tree bark was ordered from Santa Cruz Company. It was obtained in a suitable weight to be used directly. The powder was dissolved in 50 : 50 vol. methanol–dimethylformamide (DMF) solvent to prepare the stock solution. The powder was dissolved to obtain 10mg/ml concentration. It was centrifuged for 10 min. and filtered by 0.2 millipore filters. From Ellagic acid stock solution; 5 different concentrations were obtained in a serial dilution way.

The Susceptibility to Different Concentrations and Determination of Minimum Bactericidal Concentration (MBC)

In this experiment, agar diffusion technique was applied to study the antimicrobial effects of Ellagic acid against the isolates spread on Brain Heart Infusion Agar (BHI-A) medium. Ten microbial isolates were used from each type. To determine the MBC , a method conducted using a different dilutions of Ellagic acid in a range around the suspected concentrations. The MBC values were determined according to Abd-Awn *et al*, (2012).

Statistical Analyses

Processing of the data was carried out using SPSS V.25 for statistical analysis and Excel Program for Figures. Data included the calculation of mean, standard deviation (SD) and the standard error, Analyses of variance (ANOVA), and t-test were used between different groups. The confidence limit was accepted at 95% ($P=0.05$). The Probability (P -value) as follows: * $P<0.001$ High significant, ** $P<0.05$ Significant, and *** $P>0.05$ Non-Significant.

Results

Ellagic acid solution exhibited antibacterial activity against all the tested microorganisms and growth inhibition zone were formed as shown in table (1). Within the range between 10 and 0.31 mg/ml, different concentrations were used in a serial dilution using agar well diffusion assay tested on the BHI agar plates which is incubated with the tested microorganisms. The diameter of the inhibition zone found to increase as the concentrations of ellagic acid increased. The results demonstrated that ellagic acid is highly effective against all of the pathogens under the study. T- Test was used to perform comparison among different concentrations of ellagic acid solution for each pathogen and the difference was highly significant ($P<0.001$) for all the microorganisms tested as shown in table(1) and demonstrated in figure(1). Also, as demonstrated by the previously mentioned table and figure, t-test was used to perform comparison between each pathogen at the same concentration.

Table 1
Antimicrobial activity of Ellagic acid on different pathogens

Microorganism	Mean diameter of inhibition zones (mm)								<i>P</i> -value
	Control	Concentration mg/ml						No. of isolates	
		0.31	0.625	1.25	2.5	5	10		
MRSA	0.0±0.0	8.1 +0.87	11.7 +1.49	13.6 +1.34	17.1 +1.44	20 +1.82	24.2 +1.54	10	0.000**
<i>P. aeruginosa</i>	0.0±0.0	7.8 +0.78	10.8 +0.91	12.4 +0.84	16.4 +1.83	19.3 +1.25	23.4 +2.31	10	0.000**
<i>E. coli</i>	0.0±0.0	7.6 +0.69	10.4 +1.57	12.6 +1.64	16.7 +1.76	18 +0.94	23.6 +1.57	10	0.000**
<i>P</i> -value		0.366 NS	0.049*	0.096 NS	0.604 NS	0.024*	0.652 NS		

* $P<0.05$ Significant, ** $P<0.01$ High significant, NS= Non significant

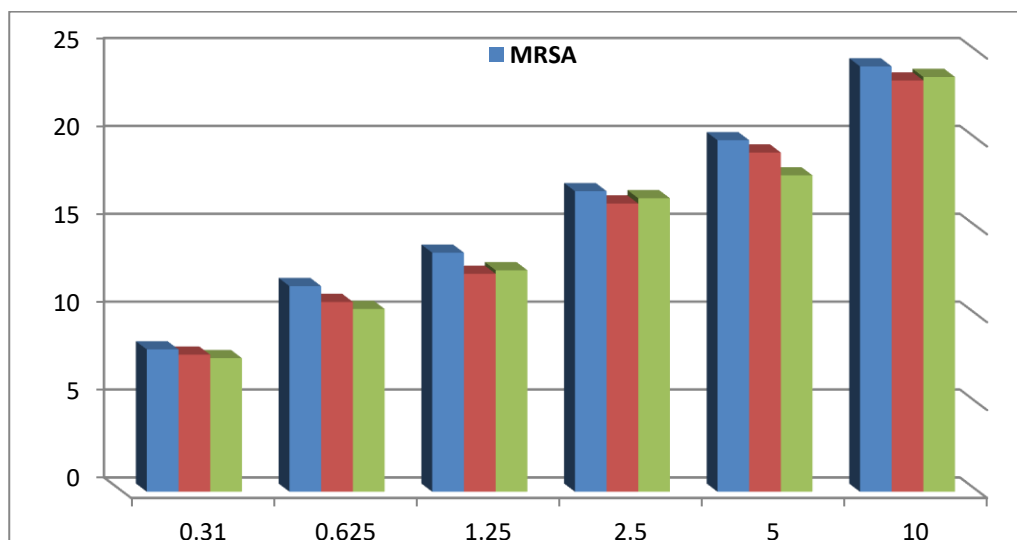


Fig. 1. Antimicrobial activity of ellagic acid on different pathogens

Ellagic acid revealed bactericidal activity against the tested bacteria. It has been found to kill the highest number of microbial isolates of MRSA, and *E. coli* at concentration of 2 mg/ml and killed the highest number of microbial isolates of *P. aeruginosa* at concentration 1.5 mg/ml, as shown in table(2). The MBC range was shown to be between (1-2) mg/ml for MRSA, and *E. coli*, and between(1-1.5) for *P. aeruginosa*.

Table 2

Number of isolates killed within the MBC for Ellagic acid on different pathogens

Microorganism	No. of isolates killed within the MBC						No. of isolates
	Concentration mg/ml					Control	
	2	1.5	1	0.5	0.25		
MRSA	5	2	3	0	0	0	10
<i>P. aeruginosa</i>	1	5	3	1	0	0	10
<i>E. coli</i>	4	3	3	0	0	0	10

Discussion

Many developing problems regarding antibiotic resistance and abuse, have increased the difficulty in treating the microbial infections nowadays. Researches continues looking for a new treatment strategies for the developing problem. Natural herbal materials consider a rich source area for discovering many compounds for many diseases treatment as well as infection counteracting. Ellagic acid which is a polyphenolic compound, present in many plant extracts, have been found to be effective against many gram-positive and gram-negative bacteria (Genskowsky *et al.*, 2016). This is also have been proved by this study. The results show that ellagic acid is highly effective against all of the microorganisms under this study. The high biological activity of ellagic acid was detected at relatively low concentrations. This deals with some previous studies

that exhibit its efficacy against many types of bacteria and fungi (Vilela *et al.*, 2017; Gullon *et al.*, 2016; Li *et al.*, 2015; loo *et al.*, 2010). Due to its poor solubility, it was challenging to find the suitable solvent to dissolve and purify it. Ellagic acid is an organic heterotetracyclic compound produced from the formal dimerisation of gallic acid by oxidative aromatic coupling with intramolecular lactonisation of both carboxylic acid groups of the resulting biaryl. It is found in many fruits and vegetables, including raspberries, strawberries, cranberries, and pomegranates. It is slightly soluble in water. In this study it was solubilized in 50 : 50 vol. methanol–dimethylformamide (DMF) solvent and purified.

A recent study develops a platform to access EA glycosides incorporating different monosaccharide residues. The study shows that the synthetic ellagic acid glycosides possess carbohydrate-specific antibiofilm effects against *Streptococcus agalactiae*. Scanning electron microscopy has revealed that these compounds likely inhibit early-stage bacterial adhesion mechanisms. These compounds additionally possess antimicrobial activity against gram-negative ESKAPE pathogens, further broadening the antibiotic profile of synthetic ellagic acid glycosides (Chambers *et al.*, 2020). In another recent study, on valonia and shell of *Q. variabilis* plants in which the ellagic acid is abundant in their extracts. The results indicated a significant antibacterial activities against *S. paratyphi* A and *S. aureus* and exhibited that antibacterial mechanism of the extracts might be by damaging the membrane of bacteria and result in great leakage of protein and nucleic acid of bacterial cells as well as the total sugar in the medium. Measurements of the SDS-PAGE of protein patterns profiles confirmed that the extract causes disruptive action on the cytoplasmic membrane. This study concluded that the cell membrane integrity is behind the inhibition of the bacterial normal growth and cellular metabolism. But it also mentioned that ellagic acid was not the main antibacterial substance in the extract and recommended a further researches on the molecular cytology level (Zhou *et al.*, 2019).

In another study, 3-O-Methyl ellagic acid derivative was found to reduce violacein production significantly and the QS-regulated biofilm formation, prodigiosin production, and protease production in *S. marcescens* (Salini and Pandian, 2015). The antimicrobial mechanisms of tannins generally can included the astringent characteristic of the tannin that may prompt complexation with enzymes or substrates which can lead to inhibition of their action. Tannin act on the membranes of the microorganisms. It can complexed with essential metal ions in bacterial cells which may account for tannins toxicity (Akiyama *et al.*, 2001; Chung *et al.*, 1998). There is a principal problem that can affect ellagic acid formulation and pharmacokinetic properties, which is its poor water solubility. Various researchers have studied an open two-compartment system with poor absorption and rapid elimination. Therefore, different formulations and systems have been formed to improve its bioavailability. For examples, an ellagic acid-phospholipid complex, a nanomedicine (thermosensitive liposomes), polymer-based nanoparticles, and nano-sized metalla-cages (Ríos *et al.*, 2018).

Inflammation and oxidative stress are related symptoms to the infections generally. Ellagic acid has been proved by previous and recent studies to exert

potent anti-inflammatory activities and antioxidative effects (Mishra and Vinayak, 2014). It's antioxidant capacity has been related to its free radical scavenging activity, which is caused by the presence of four hydroxyl and two lactone functional groups which enable ellagic acid to scavenge an extensive variety of ROS, such as hydroxyl, hydroperoxyl, and peroxy radicals, as well as nitrogen dioxide and peroxyxynitrite. The anti-inflammatory effect has been related to various mechanisms, such as the decrease in pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α), the increase of anti-inflammatory cytokines (IL-10), and the inhibition of transcription factors (AP-1) and various kinases (MAPK, ERK1/2, JNK) (Corn lio *et al.*, 2013; Gonz lez *et al.*, 2010). Secretion of pro-inflammatory mediators such as MIF and MCP-1 was also reported, as well as the reduction of TLR2 and TLR4 protein levels and mRNA expression in liver tissues (Lee *et al.*, 2014). In an *in vivo* animal study, administration of ellagic acid to mice showed neither acute toxicity nor chronic effects even up to 5000 mg/kg (Reddy *et al.*, 2014). Also, doses of 100 mg/kg/d administered intra peritoneally exerted no toxicity in mice (Soh *et al.*, 2009). The experimental data with the limited results, to date, has drawn a great amount of interest in this compound.

Conclusion

This current study has detected and proved the antimicrobial activity of ellagic acid against important and widely resistant pathogenic bacteria like MRSA, *P. aeruginosa*, and *E.coli*. Further work should be performed on ellagic acid to be used and utilized in pharmaceutical application as a therapeutic drug.

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